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IRF2-binding protein-1 is a JDP2 ubiquitin ligase and an inhibitor of ATF2-dependent transcription

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Abstract Jun-dimerization protein 2 (JDP2) is a member of the activating protein-1 (AP-1) family of transcription factors. JDP2 dimerizes with other AP-1 proteins such as activating transcription factor-2 (ATF2) and Jun to repress transcription from promoters that contain a cyclic AMP-responsive element (CRE). Interferon regulatory factor-2-binding protein-1 (IRF2-BP1), which is reported to be a transcriptional corepressor of IRF2, was isolated as a JDP2-binding protein using an epitope-tagging method. As anticipated from the presence of a RING-finger domain, IRF2-BP1 enhanced the polyubiquitination of JDP2. Moreover, IRF2-BP1 repressed ATF2-mediated transcriptional activation from a CRE-containing promoter.

Structured summary:
MINT-6699624:
JDP2 (uniprotkb:Q8WYK2) *physically interacts* (MI:0218) with *Valyl-tRNA synthetase* (uniprotkb:P26640), *IRF2-BP1* (uniprotkb:Q8IU81), *ATF7* (uniprotkb:P17544), *EEF1G* (uniprotkb:P26641), *EEF1A1* (uniprotkb:P68104), *JUND* (uniprotkb:P17535), *JUNB* (uniprotkb:P17275), *EF1D2* (uniprotkb:P29692) and *RPLP0* (uniprotkb:P05388) by *anti tag coimmunoprecipitation* (MI:0007).
MINT-6699850:
JDP2 (uniprotkb:Q8WYK2) *binds* (MI:0407) to *IRF2-BP1* (uniprotkb:Q8IU81) by *pull down* (MI:0096).
MINT-6699684:
JDP2 (uniprotkb:Q8WYK2) *physically interacts* (MI:0218) with *JUNB* (uniprotkb:P17275), *JUND* (uniprotkb:P17535), *ATF7* (uniprotkb:P17544) and *IRF2-BP1* (uniprotkb:Q8IU81) by *anti tag coimmunoprecipitation* (MI:0007).
MINT-6699839:
IRF2-BP1 (uniprotkb:Q8IU81) *physically interacts* (MI:0218) with *JDP2* (uniprotkb:Q8WYK2) by *anti bait coimmunoprecipitation* (MI:0006).
MINT-6699748:
JDP2 (uniprotkb:Q8WYK2) *physically interacts* (MI:0218) with *ATF2* (uniprotkb:P15336) and *c-JUN* (uniprotkb:P05412) by *anti tag coimmunoprecipitation* (MI:0007).
MINT-6699865:
ATF2 (uniprotkb:P15336) *binds* (MI:0407) to *IRF2-BP1* (uniprotkb:Q8IU81) by *pull down* (MI:0096).

Keywords: AP-1; ATF2; IRF2; JDP2 RING E3 ligase; Transcriptional repression

1. Introduction

Activating protein-1 (AP-1) proteins are transcription factors that are composed of hetero- or homodimers of basic leucine zipper proteins, many of which belong to the Jun, Fos, or activating transcription factor (ATF) families. These proteins control cell proliferation and differentiation by regulating the expression of cell cycle-related genes and function in both pro- and anti-apoptotic processes [1]. In addition, AP-1 proteins play roles in the induction of interferon-β; the incorporation of the ATF2/c-Jun heterodimer into a complex with interferon regulatory factors (IRFs) is essential for the transcriptional activation [2].

Jun-dimerization protein (JDP2), another AP-1 protein, is a dimerization partner of Jun [3] and ATF2 [4] and represses transcriptional activation. JDP2 has effects on various cellular processes. It mediates the differentiation of myoblasts [5] and osteoclasts [6], inhibits the differentiation of adipocytes [7] and embryonic carcinoma cells [8], acts as a tumor suppressor in NIH3T3 cells and prostate cancer cell lines [9], induces the partial transformation of chick embryonic fibroblasts [10], and functions as a cell-survival protein [11] and as a progesterone receptor coactivator [12]. The mechanisms of JDP2-mediated transcriptional repression may include the regulation of nucleosome assembly and histone acetylation [13]. These diverse functions of JDP2 suggest that it cooperates with various proteins to mediate regulatory activity.

2. Materials and methods

2.1. Plasmids

Human *IRF2-BP1* cDNA was obtained from the American Type Culture Collection (ATCC number 9115061); *JDP2* and *ATF2* cDNA was isolated from HeLa cells using PCR. The plasmids pIRES-JDP2 and pIRES-f-JDP2 contain untagged and N-terminally FLAG-tagged *JDP2* cDNA, respectively, within pIRESneo3 (Clontech). The pcDNA-IRF2BP1, pcDNA-ATF2, pcDNA-JDP2, and

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Abbreviations: AP-1, activating protein-1; ATF, activating transcription factor; CRE, cyclic AMP-responsive element; DTT, dithiothreitol; HA, hemagglutinin; IRF, interferon regulatory factor; IRF2-BP1, IRF2-binding protein-1; JDP2, Jun-dimerization protein 2; PMSF, phenylmethylsulfonyl fluoride

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pcDNA-JDP2-His carry the corresponding cDNA within pcDNA3 (Invitrogen). The pcDNA-IRF2BP1ΔR and pcDNA-IRF2BP1sub contain either a termination codon after position 501 or Cys527Ser and Cys530Ser mutations, respectively, within the *IRF2-BP1* open reading frame. The phRL-3CRE-TK (RIKEN DNA bank number 3459) is a derivative of phRL-TK (Promega) that has three cyclic AMP-responsive elements (CREs) inserted upstream of the promoter. The pMT123 encodes a hemagglutinin (HA)-tagged precursor of ubiquitin [14].

2.2. Cells

HeLa-S3, HeLa-G, and HepG2 cells were obtained from the RIKEN Cell Bank. To establish the stable transformant lines HeLa/JDP2 and HeLa/f-JDP2, HeLa-S3 cells were transfected with pIRES-JDP2 and pIRES-f-JDP2, respectively, and cloned.

2.3. Antibodies

Antibodies against IRF2-binding protein-1 (IRF2-BP1) and JDP2 were raised in rabbits immunized with bacterially expressed His_6-tagged IRF2-BP1 and JDP2 proteins, respectively. Other polyclonal antibodies were obtained from Santa Cruz Biotechnology. FLAG-specific (M2) and HA-specific (12CA5) monoclonal antibodies were obtained from Sigma and Roche, respectively.

2.4. Isolation and identification of JDP2-binding proteins

Nuclear extracts were prepared from HeLa/JDP2 and HeLa/f-JDP2 cells as described [15]. After the extracts were prepared in BC buffer [20 mM Tris–HCl (pH 8.0 at 4 °C), 20% glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT)] containing 100 mM KCl and 0.5% Nonidet P-40, the FLAG-tagged protein complexes were collected with M2 antibody-conjugated agarose (Sigma) in a batch procedure over 4 h at 4 °C. The M2-agarose was then washed four times with BC buffer containing 300 mM KCl and 0.5% Nonidet P-40, and the proteins were eluted with BC buffer containing 100 mM NaCl, 0.1% Nonidet P-40, and 200 μg/ml FLAG-peptide (Sigma). Following SDS–PAGE, JDP2-binding proteins were subjected to in-gel trypsin digestion. The molecular masses of the eluted peptides were determined using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Shimadzu) and searched against a database using the MS-Fit program (University of California, San Francisco).

2.5. Immunoprecipitation

A nuclear extract of HeLa/f-JDP2 cells was prepared in nuclear buffer [420 mM NaCl, 20 mM Tris–HCl (pH 8.0 at 4 °C), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, 2 mM DTT] [15] and precleared with protein A-Sepharose (GE Healthcare). Antibodies conjugated to protein A-Sepharose were added to the precleared nuclear extract, and the mixture was rocked for 4 h at 4 °C. After four washes with BC buffer containing 400 mM KCl and 0.5% Nonidet P-40, proteins bound to the protein A-Sepharose were eluted with SDS–PAGE sample buffer.

2.6. Glutathione S-transferase (GST) pull-down assay

The GST fusion proteins GST-JDP2, GST-ATF2, and GST were expressed in *Escherichia coli* and purified using glutathione-Sepharose (GE Healthcare). The ^{35}S-labeled IRF2-BP1 was prepared by in vitro transcription and translation using the TNT-Coupled Reticulocyte Lysate System (Promega). The GST fusion proteins were bound to glutathione-Sepharose and then mixed with the in vitro-translation products in binding buffer [200 mM NaCl, 20 mM Tris–HCl (pH 8.0 at 4 °C), 20% glycerol, 1 mM EDTA, 0.5 mM PMSF, 0.5% Nonidet P-40]. After rocking for 2 h at 4 °C and four washes, the proteins bound to glutathione-sepharose were eluted with SDS–PAGE sample buffer. The radioactivity in the SDS–PAGE gel was visualized using a BAS2500 image analyzer (Fuji).

2.7. Ubiquitination assay

HeLa-G cells in 10-cm dishes were co-transfected with plasmids (Fig. 3). The pMT123, pcDNA-JDP2-His, pcDNA-IRF2BP1, pcDNA-IRF2BP1ΔR, pcDNA-IRF2BP1sub, and pcDNA3 (8 μg each) were used for transfection. After a 24-h incubation, MG132 (Peptide Institute) was added to a final concentration of 5 μM, and the cells were incubated for another 4 h. The cells were washed and lysed in denaturation buffer [6 M guanidine–HCl, 100 mM Tris–HCl (pH 8.0 at 4 °C), 5 mM imidazole]. The His_6-tagged JDP2 was purified from the denatured cell lysate using Ni^{2+}-nitrilotriacetic acid agarose (Qiagen) as described [14] and analyzed by Western blotting with antibodies specific to the HA-tag or JDP2. Signals were visualized by chemiluminescence and quantified with a LuminoImage analyzer Las-3000 (Fuji).

2.8. Luciferase assay

HepG2 cells were seeded in a 96-well plate at 6×10^4 cells/well and incubated for 24 h. The cells were then co-transfected with plasmid (Fig. 4). After incubating for 29 h, luminescence was measured using the *Renilla* Luciferase Assay System (Promega) and an ARVO Light Luminescence Counter (Perkin–Elmer). The results are provided as the mean ± S.E. ($n = 4$) relative to the value for the reporter alone, which was defined as 1.0.

3. Results

3.1. IRF2-BP1 associates with JDP2

FLAG-JDP2 and associated proteins were isolated from nuclear extracts of the stably transformed cell lines HeLa/JDP2 and HeLa/f-JDP2, which express untagged and FLAG-tagged JDP2, respectively, using the FLAG-specific M2 antibody. The proteins that were recovered from HeLa/f-JDP2, but not from HeLa/JDP2, were identified using peptide mass fingerprinting (Fig. 1A and Supplementary Fig. S1). The identification of transcription-related proteins was confirmed by Western blotting (Fig. 1B). Interactions between JDP2 and ATF7 or IRF2-BP1 have not been reported previously.

Translation factors were also among the isolated proteins (Fig. 1A and Supplementary Fig. S1). The isolation of JunB and JunD, which are confirmed partners of JDP2, supports the hypothesis that these proteins are associated with JDP2. The formation of a complex of valyl-tRNA synthetase and EF-1 proteins has been reported [16].

Because we did not find certain proteins that were previously reported to interact with JDP2, their possible presence in the isolated JDP2 complexes was examined by Western blotting (Fig. 1C). Both *c*-Jun and ATF2 were detected in the complexes, but neither histone deacetylase-3 nor p300 was detected.

The interaction between IRF2-BP1 and JDP2 was confirmed. FLAG-JDP2 was precipitated from the HeLa/f-JDP2 nuclear extract using IRF2-BP1-specific antiserum, but not preimmune serum (Fig. 2A). In addition, in vitro-translated IRF2-BP1 was bound to GST-JDP2, but not to GST (Fig. 2B).

3.2. IRF2-BP1 enhances the ubiquitination of JDP2

A region in the C-terminal portion of IRF2-BP1 is approximately 90% homologous to the RING-finger domain of KIAA1865 (Fig. 3A), for which the three-dimensional structure has been solved by NMR (Protein Data Bank number 2cs3). Thus, this region in IRF2-BP1 is reasonably assumed to be a RING-finger domain.

Because many RING-finger proteins show ubiquitin ligase E3 activity [17], the possible ubiquitination of JDP2 by IRF2-BP1 was examined. His_6-tagged JDP2, HA-tagged ubiquitin precursor, and IRF2-BP1 were expressed in cultured cells, and the ubiquitination of the purified JDP2-His was

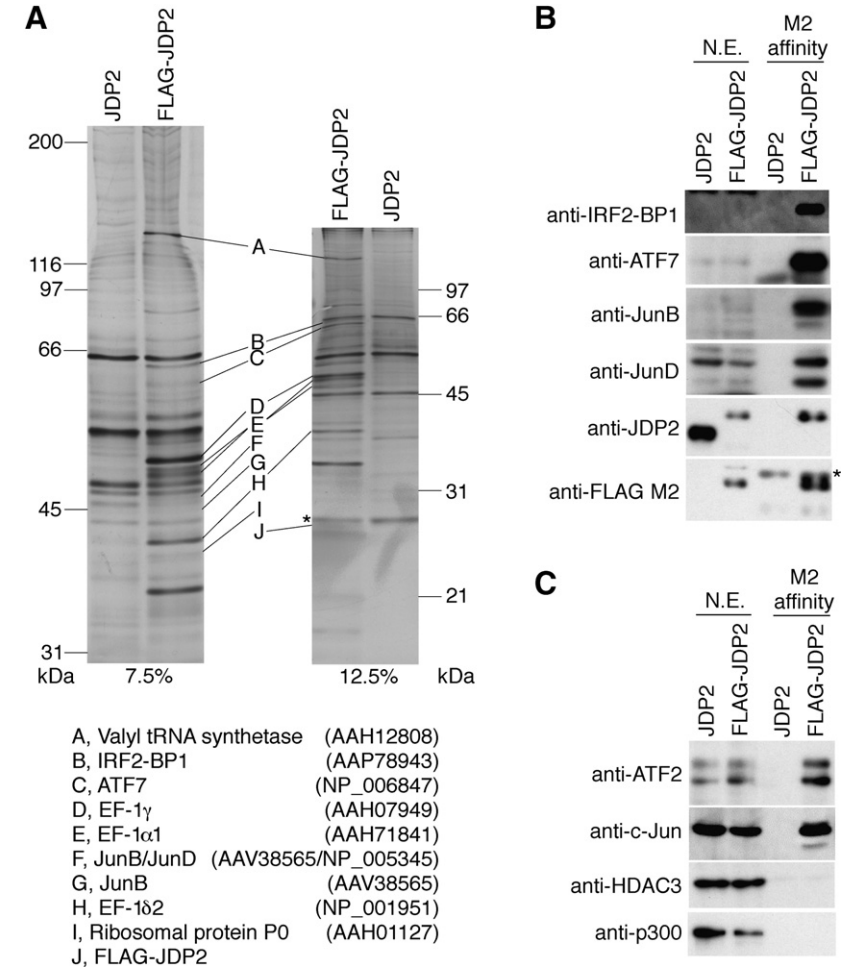

Fig. 1. (A) JDP2-binding proteins were isolated from nuclear extracts of HeLa/JDP2 (JDP2) and HeLa/f-JDP2 (FLAG-JDP2) cells using FLAG-specific M2 antibody and electrophoresed on 7.5% and 12.5% polyacrylamide gels. The proteins identified by peptide mass fingerprinting are denoted with accession numbers. EF-1α1 was present in the pair of bands. Both JunB and JunD were identified in band F. (B) Nuclear extracts (N.E.) and M2-affinity-purified fractions from each cell line were subjected to Western blotting with specific antibodies. Certain proteins were concentrated in the M2-affinity-purified fractions. FLAG-JDP2 migrated more slowly than untagged JDP2 during SDS–PAGE. No endogenous JDP2 was detected. The asterisk denotes the light chain of the M2 antibody. (C) The same fractions were subjected to Western blotting with antibodies specific to proteins that were expected to interact with JDP2 but were not identified in (A).

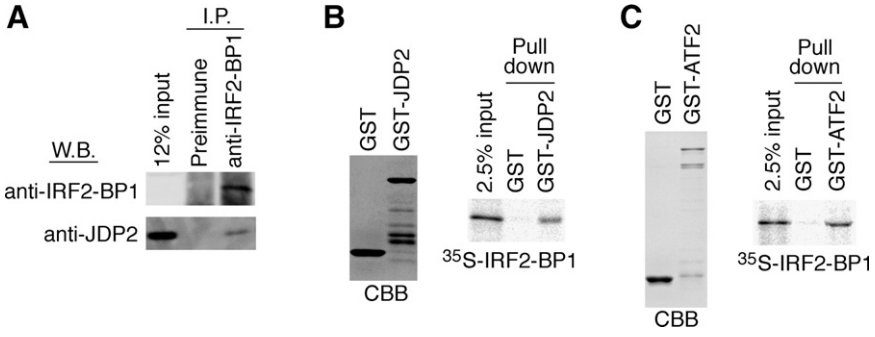

Fig. 2. (A) Fractions immunoprecipitated (I.P.) from a HeLa/f-JDP2 nuclear extract with IRF2-BP1-specific or preimmune antibody were probed with anti-IRF2-BP1 and anti-JDP2 antibodies by Western blotting (W.B.). (B and C) (Left) Coomassie Brilliant Blue (CBB) staining of the used proteins. (Right) ^{35}S-labeled IRF2-BP1 translated in vitro was used in a GST pull-down assay. The input and bound (Pull down) fractions were subjected to SDS–PAGE and images of the gels were collected by an imaging plate.

examined using an HA-specific antibody. Bands that were immunoreactive to the HA-specific antibody (Fig. 3B, upper panel) could be attributed to poly-HA-ubiquitinated JDP2-His because cells expressing either JDP2-His or HA-ubiquitin

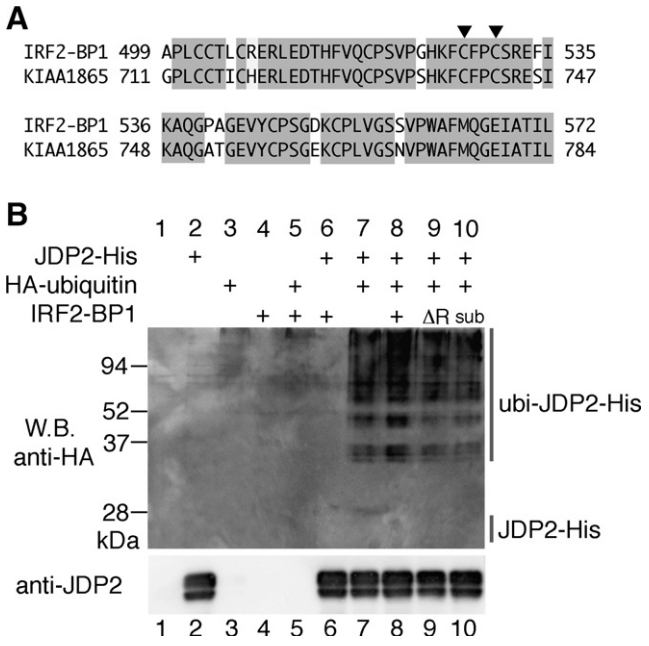

Fig. 3. (A) Sequence alignment of the RING-finger domains of IRF2-BP1 (AAP78943) and KIAA1865 (BAB47494). Identical residues are shaded and amino acid positions are indicated. Triangles indicate Cys residues substituted by Ser residues in the IRF2-BP1sub mutant. (B) HeLa-G cells were co-transfected with expression plasmids for JDP2-His, HA-ubiquitin precursor, or IRF2-BP1 in the indicated combinations. Lanes 9 and 10 contain plasmids expressing IRF2-BP1ΔR and IRF2-BP1sub, respectively, in place of the wild-type IRF2-BP1. The ubiquitination of JDP2-His was analyzed as described in the text.

alone did not show these bands (lanes 2–6). When IRF2-BP1 was co-expressed with both JDP2-His and HA-ubiquitin, the level of JDP2-His did not change (lanes 7 and 8, lower panel), but the intensity of the polyubiquitination was enhanced 1.84 ± 0.48-fold (upper panel) in three independent experiments. The expression of IRF2-BP1 ΔR (lane 9), which lacks the RING-finger domain, and of IRF2-BP1sub (lane 10), which has two Cys-to-Ser substitutions within its RING-finger domain (Fig. 3A), did not increase the ubiquitination of JDP2.

3.3. IRF2-BP1 represses ATF2-dependent transcriptional activation

The effects of IRF2-BP1 on ATF2- and JDP2-mediated transcriptional regulation were examined using a luciferase assay system. ATF2-dependent activation of a CRE-containing promoter and dose-dependent inhibition of it by JDP2 were observed (Fig. 4A). IRF2-BP1 repressed ATF2-dependent activation in a dose-dependent manner in the absence of plasmid-encoded JDP2 (Fig. 4B, columns 2–5). When JDP2 was expressed at a level that partially inhibited ATF2-dependent activation (column 9), IRF2-BP1 still repressed transcription (columns 10–12), and the relative extents of repression were similar to those in the absence of JDP2 (columns 3–5). Therefore, the effects of IRF2BP1 and JDP2 on the ATF2-dependent activation are additive.

The repression by IRF2-BP1 observed in the absence of plasmid-encoded JDP2 raised the possibility that IRF2-BP1 might interact directly with ATF2. IRF2-BP1 was pulled down by GST-ATF2, but not by GST (Fig. 2C), indicating that ATF2 and IRF2-BP1 can interact directly. No IRF2-BP1-dependent ubiquitination of ATF2 was observed (data not shown).

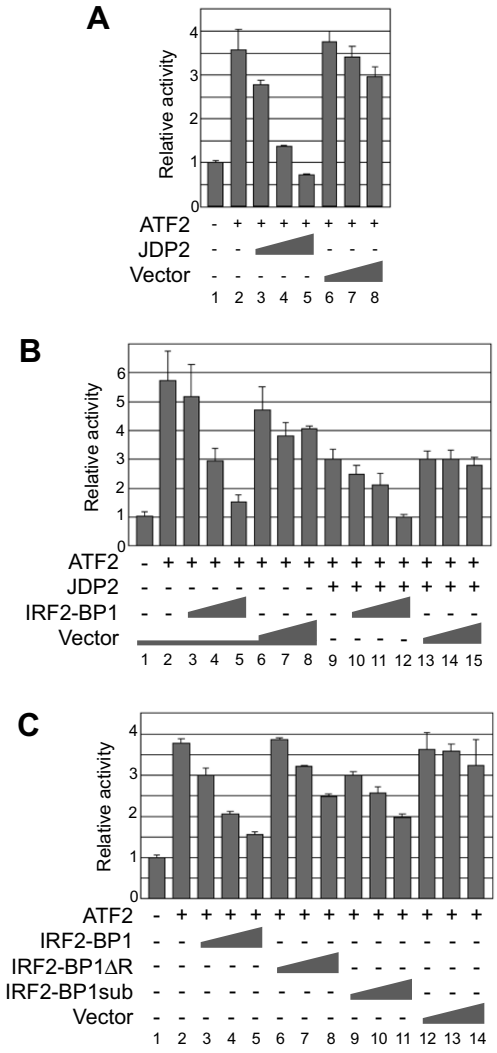

Fig. 4. Luciferase assay. The phRL-3CRE-TK reporter plasmid (50 ng/well), pcDNA-ATF2 (50 ng/well), and varying amounts of pcDNA-JDP2, pcDNA-IRF2BP1, and pcDNA3 (Vector) were co-transfected. Incremental shading indicates 6.3, 25, and 100 ng/well of the plasmids. The amounts of DNA were equalized by the addition of pUC19. (A) JDP2 represses ATF2-dependent activation. (B) IRF2-BP1 represses ATF2-dependent activation in the presence and absence of JDP2. The amounts of pcDNA-JDP2 (6.3 ng/well) in columns 9–15 were balanced in columns 1–8 by the vector (shaded horizontal bar). (C) The ubiquitin ligase activity of IRF2-BP1 is not essential for transcriptional repression. IRF2BP1ΔR or IRF2BP1sub, both lacking ubiquitin ligase activity, were expressed.

The relationship between the ubiquitin ligase and transcriptional inhibitor activities of IRF2-BP1 was addressed. Activation was repressed when either IRF2-BP1ΔR or IRF2-BP1sub, which both lack ligase activity (Fig. 3B), was co-expressed with ATF2 (Fig. 4C, columns 6–11). Thus, the repression occurred independently of ubiquitin ligase activity.

4. Discussion

IRF2-BP1 was originally reported to be a corepressor for IRF2 [18], which is a repressor of the interferon-β gene.

IRF2 competes with IRF1, an activator of the gene, to bind to the same regulatory elements [19]. Importantly, the ATF2/*c*-Jun heterodimer binds to a site adjacent to the IRF-binding elements during the induction of interferon-β [2]. Because JDP2 represses activation by ATF2 and *c*-Jun, IRF2-BP1 might interact with both IRF2 and JDP2 as a corepressor.

Polyubiquitination of a protein is generally a prelude to degradation. The two functions of IRF2-BP1, i.e., repression and ubiquitination, might therefore be in conflict. However, ubiquitin ligase activity is not required for transcriptional repression. Hence, it is possible that in the repressor complex, IRF2-BP1 does not ubiquitinate JDP2, whereas under some circumstances, it is activated to ubiquitinate JDP2, leading to JDP2 degradation and transcriptional activation. To support this hypothesis, the participation of a ubiquitin-specific protease like HAUSP for p53 [20] and USP28 for Myc [21] can be postulated.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.07.033.

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